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Proteoliposome-based selection of a recombinant antibody fragment against the human M2 muscarinic acetylcholine receptor

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Abstract

The development of antibodies against human G-protein-coupled receptors (GPCRs) has achieved limited success, and this has mainly been attributed to their low stability in a detergent-solubilized state. We herein described a method that can generally be applied to the selection of phage display libraries with human GPCRs reconstituted in liposomes. A key feature of this approach is the production of biotinylated proteoliposomes that can be immobilized on the surface of streptavidin-coupled microplates or paramagnetic beads and used as a binding target for antibodies. As an example, we isolated a single chain Fv fragment from an immune phage library that specifically binds to the human M2 muscarinic acetylcholine receptor with nanomolar affinity. The selected antibody fragment recognized the GPCR in both detergent-solubilized and in membrane-embedded forms, which suggested that it may be a potentially valuable tool for structural and functional studies of the GPCR. The use of proteoliposomes as immunogens and screening bait will facilitate the application of phage display to this difficult class of membrane proteins.

Keywords

Phage display; Antibody fragment; GPCR; Proteoliposome; Screening

Introduction

G-protein-coupled receptors (GPCRs) comprise the largest class of signal-transducing receptors involved in a broad range of physiological processes ranging from cell cycle control to metabolism and the actions of hormones.^(1,2) GPCR dysfunctions have been implicated in various pathological processes including cardiovascular, gastrointestinal, metabolic, neurodegenerative, psychiatric, and immune disorders as well as cancers. More than 30% of all clinically approved therapeutics currently target GPCRs.⁽³⁾ As understanding of GPCR-associated disease pathologies increases, the need for new antibodies related to receptor characterization, purification, tissue localization, clinical diagnostics, and therapeutics is also growing.

However, raising antibodies against GPCRs is technically challenging for the following reasons: (i) the poor immunogenicity of GPCRs, which are largely buried in the membrane, (ii) the high degree of sequence homology between human and mouse genes, (iii) the low density of GPCRs in native cell membranes, (iv) the difficulties associated with obtaining sufficient amounts of the purified GPCRs expressed in heterologous hosts, and (v) most GPCRs in detergent-solubilized state are unstable and are likely to aggregate easily, such that denatured proteins and aggregation may cause non-specific binding during the selection of antibodies, thereby producing false positives. Furthermore, antibodies against GPCR-derived peptides rarely recognize native receptors and have adequate affinities or specificities, because the linear peptide does not necessarily replicate the loop in the complete GPCR structure. An approach

using purified human GPCRs in reconstituted liposomes as binding targets may overcome these problems.

Phage display technology offers an attractive strategy to facilitate the isolation of novel antibody fragments. The process is easier, faster, and less labor intensive than traditional hybridoma technology, in which significant expertise and time-consuming cell culture steps are needed to accomplish the desired antibody selection. Antibody phage libraries have been constructed under various settings, including naïve, synthetic, and immunized libraries.⁽⁴⁾ Most high-quality naïve and synthetic libraries are proprietary or not yet commercially available, and it is also difficult to maintain highly complex diversity (10^9 - 10^{12} independent clones) during propagation in a routine laboratory.^(5,6) In contrast, the construction and selection of immune libraries is a promising starting point because a smaller library size (5×10^5 - 1×10^6 clones) was previously shown to be sufficient for isolating high affinity binders.^(7,8) Consecutive rounds of immunization and *in vivo* affinity maturation by the murine immune system is likely to result in a high frequency of antigen-specific B-cells, producing large amounts of antibodies and the corresponding mRNAs used for the generation of the focused library.

The M2 muscarinic acetylcholine receptor (M2 receptor) is a GPCR that has an essential role in the physiological control of cardiovascular function and many pivotal central processes, such as cognition and pain perception.⁽⁹⁾ Due to its importance in medical and basic biological research, we selected the human M2 receptor as a model target in this study. We here demonstrated the isolation of a single chain Fv (scFv)

fragment against the human M2 receptor using a proteoliposome-targeted strategy, in both animal immunization and the subsequent selection of an immune phage display library. We also described simple and reliable methods used to characterize the binding properties of the selected antibody.

Materials and methods

Proteoliposome antigen preparation

A variant of the human M2 receptor, M2-i3d, which lacks the central part of the third intracellular loop (ICL3) from Ser234 to Arg381 as well as the native glycosylation sites, was expressed in Sf9 insect cells, as described previously.⁽¹⁰⁾ After solubilizing the membrane with digitonin/Na-cholate solution, M2-i3d bound to the high-affinity inverse agonist 3-quinuclidinyl-benzilate (QNB) was purified by using an aminobenzotropine (ABT) affinity column and hydroxyapatite column, as described.⁽¹¹⁾ The eluate was concentrated with Amicon Ultra (Merck Millipore, Billerica, MA), and dialyzed against 20 mM HEPES-NaOH, pH 7.5, 200 mM NaCl, 5% glycerol, 0.05% n-dodecyl- β -D-maltopyranoside (DDM) (Anatrace, Maumee, OH), and 0.01% cholesterol hemisuccinate (CHS) (Sigma-Aldrich, St. Louis, MO). Proteoliposomes were prepared by removing the detergent from mixed lipid/detergent micelles using Bio-Beads SM-2 (Bio-Rad, Hercules, CA). Briefly, 1 mg of purified QNB-bound M2-i3d was added to a mixture of 4 mg of egg yolk phosphatidylcholine (egg PC; Avanti Polar Lipids, Alabaster, AL) and 1 mg of adjuvant Lipid A (Sigma-Aldrich) in 1 mL of PBS containing 0.8% sodium cholate (Dojindo, Kumamoto, Japan). This was immediately followed by the addition of fresh Bio-Beads SM-2 and overnight incubation at 4°C. After removing the beads, the resultant suspensions were sonicated to produce small unilamellar vesicles. The reconstituted proteoliposome was stored at -80°C prior to use for immunization.

Immunization and antibody library construction

All animal experiments described in this study conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals of Japan and were approved by the University of Tokyo Animal Care Committee (approval no. RAC07101). M2 and M4 receptor double knockout mice were initially immunized with 0.1 mg of the proteoliposome antigen and 0.1 μ g of pertussis toxin, followed by two or three booster injections of 0.1 mg of the proteoliposome antigen at two-week intervals. Immunized mice were sacrificed, and total RNA in the spleen was isolated using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using an oligo (dT) primer and SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham MA) in a standard procedure.⁽¹²⁾ A combinatorial single-chain Fv (scFv) antibody library was generated by PCR amplification and the assembly of V_L and V_H immunoglobulin domains with an 18 amino acid flexible linker, and was then cloned into the phagemid vector pComb3XSS, as described.⁽¹³⁾

Proteoliposome-targeted panning of the combinatorial scFv library

Biotinylated proteoliposomes were used as the target for selection, and were prepared by the same method as that for the proteoliposome antigen described above, except that 0.5 mg/mL of the membrane protein was reconstituted with a mixture of 5 mg/mL of egg PC and 25 μ g/mL of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (16:0 biotinyl

Cap-PE; Avanti Polar Lipids). To perform negative selection steps (i.e., subtractive panning on an otherwise equivalent system that lacks the target membrane protein), “empty” biotinylated liposomes were prepared with egg PC and 16:0 biotinyl Cap-PE.

All steps of the phage display selection were performed at 4°C or on ice. In the first selection cycle, 2×10^{13} phage particles displaying the scFv library (1 mL) were incubated for 1 h with “empty” biotinylated liposomes (200 μ L; 50 μ g/mL lipid in TBS-B buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA]). Phage-empty liposome complexes were captured on 100 μ L streptavidin-coated paramagnetic beads (10 mg/mL, Dynabeads MyOne Streptavidin T1, Thermo Fisher Scientific) for 1 h. The supernatant (1 mL) was collected and incubated with the biotinylated proteoliposome bait (200 μ L; 50 μ g/mL lipid and 5 μ g/mL membrane protein in TBS-B) for 1 h. The phage-proteoliposome complexes were captured on the beads for 30 min. After washing the beads eight times with TBS-B, the phage particles were eluted with 100 μ L of 100 mM glycine-HCl, pH 2.5, for 10 min. Eluates were neutralized with 5 μ L of 1 M Tris-HCl, pH 9.0, and used to infect 2 mL of exponentially growing *E.coli* XL-1 Blue cells (Agilent Technologies, Santa Clara, CA). After shaking for 1 h at 37°C, cells were plated on LB agar plates containing 100 μ g/mL ampicillin and grown overnight at 37°C. The cells were scraped off the plates, and phage rescue was performed using the VCSM13 helper phage (Agilent Technologies) according to a standard method described previously.⁽¹⁴⁾

In the subsequent selection rounds, 10^{12} of the amplified phage particles were used as an input, and beads were washed 10 to 15 times with TBS-B. After the fourth round,

single clones were randomly picked and analyzed by a proteoliposome-targeted enzyme-linked immunosorbent assay (liposome ELISA; Fig. 1A) as described below, and the positive clones were sequenced.

Liposome ELISA

E. coli XL-1 Blue, harboring pComb3XSS encoding a selected scFv, was grown overnight at 37°C. Two milliliters of SB (20 g of tryptone, 10 g of yeast extract, 5 g of NaCl per liter) containing 100 µg/mL ampicillin was inoculated with 50 µL of the overnight culture. After being incubated for 3 h at 37°C, expression was induced with IPTG (1 mM final concentration) and cells were grown at 30°C overnight with vigorous shaking. Cells were harvested, and the periplasmic extract was prepared by suspending the cells in an ice-cold high osmotic buffer (50 mM HEPES-NaOH, pH 7.5, 0.5 mM EDTA, 20% sucrose), followed by the addition of lysozyme (3 mg/mL final concentration) and incubation for 30 min at 37°C. The bacterial cells were removed by centrifugation at 15,000 x g for 10 min, and the scFv-containing supernatant was used for liposome ELISA, Biacore analysis, and epitope mapping. The scFv had a HA tag at the C-terminus.

Biotinylated proteoliposome bait (25 µL; 200 µg/mL lipid and 20 µg/mL membrane protein in TBS-B) or biotinylated empty liposomes (25 µL; 200 µg/mL lipid in TBS-B) were mixed with 100 µL of the *E. coli* periplasmic extract and incubated for 1 h on ice. The scFv-liposome complex was applied to a well of a Nunc Immobilizer Streptavidin plate (Thermo Fisher Scientific) and further incubated for 30 min on ice. After

extensive washing with TBS, bound scFv was detected with the anti-HA antibody POD conjugate (Roche Diagnostics, Basel, Switzerland; 1:1000 dilution in TBS-B, 1 h on ice) using ABTS (Sigma-Aldrich) as a substrate for POD. The absorbance of the color reaction was measured at 415 nm with a microplate reader (model 680, Bio-Rad).

Binding kinetics measurements

Surface plasmon resonance (SPR) experiments were performed at 20°C using the Biacore T100 with series S streptavidin sensor chips (Series S sensor chip SA; GE Healthcare, Little Chalfont, UK) in the running buffer containing 20 mM HEPES-NaOH, pH 7.5, 200 mM NaCl, 5% glycerol, 0.05% DDM, and 0.01% CHS. Biotinylated anti-HA IgG (Rockland Immunochemicals, Gilbertsville, PA) was captured on the streptavidin surface (flow cells 1 and 2) by a 400-sec injection of 50 µg/mL of the antibody at 10 µL/min. Crude scFv-containing *E. coli* periplasmic extracts with 12 mg/ml BSA and 12 mg/ml CM-dextran were subsequently injected (500 µL at a flow rate of 10 µL/min) over flow cell 2, in which the C-terminal HA-tagged scFvs were captured. QNB-bound M2-i3d at various concentrations was simultaneously passed over flow cells 1 and 2 at a flow rate of 30 µL/min. Association and dissociation times were 2 and 5 min, respectively, and regeneration consisted of a 30-sec injection of 10 mM NaOH. Flow cell 1 was used as a reference surface. Data analysis was performed using BIAevaluation software (GE Healthcare).

Epitope mapping

The variants used for epitope mapping on M2-i3d are summarized in Table 1. Site-directed mutagenesis was conducted following a PCR-based protocol⁽¹⁵⁾ or *in vivo* homologous recombination in *Saccharomyces cerevisiae*.⁽¹⁶⁾ Different DNA fragments encoding M2-i3d and its variants were inserted into the vector pDDGFP-2,⁽¹⁷⁾ and all the expression constructs were verified by DNA sequencing of the entire gene through the ligation junctions with the vector plasmid. The resultant constructs with a C-terminal fusion to GFP were overexpressed in *S. cerevisiae*, and membranes from small-scale (10 mL) cultures were prepared by the mechanical disruption of cells, which was achieved by shaking with glass beads, as described.⁽¹⁸⁾

A Nunc Maxisorp plate (Thermo Fisher Scientific) was coated with the membrane fractions containing an equivalent amount of M2-i3d and its variants in PBS, pH 7.4, at 4°C overnight and blocked for 2 h with PBS-B (PBS, pH 7.4, containing 1% BSA) at 4°C. The wells were incubated with 100 µL of HA-tagged scFv (10 µg/mL) in PBS-B for 1 h at 4°C. The bound HA-tagged scFv was detected as described above.

Results

Library construction and selection

M2-i3d was used as a binding target throughout the present study because the central part of ICL3 in the human M2 receptor can be removed without impairing its ability to bind to agonists or activate G proteins.⁽¹⁹⁾ Functional and folded proteins were specifically retrieved by ABT affinity column chromatography, and the purified M2-i3d used in proteoliposome reconstitution was in an inactive antagonist-bound conformation.

Among the five different muscarinic receptors, subtypes M1-M5, that have been cloned and characterized, the primary sequences of subtypes M2 and M4 are more homologous to each other than to other subtypes. In addition, the M2 and M4 receptors both couple efficiently to inhibit cyclic AMP,⁽²⁰⁾ suggesting similarities in the functional tertiary structures of the two subtypes. To abrogate the mechanisms of immune tolerance, two M2/M4 double knockout mice were immunized with M2-i3d-reconstituted liposomes to generate a library of murine immunoglobulin genes. To facilitate the immune response, adjuvant Lipid A was added as an ingredient to the proteoliposomes.⁽²¹⁾ The sera obtained from mice became immunoreactive after the third or fourth immunization, as indicated by liposome ELISA (data not shown). Animals were sacrificed three days after the last immunization, and a scFv phage library was prepared. The library consisted of approximately 2.9×10^8 independent transformants.

Liposomes were immobilized via a biotinylated lipid on streptavidin-coupled

paramagnetic beads for phage panning. Four rounds of subtractive screening were performed using M2-i3d-proteoliposomes and empty liposomes devoid of M2-i3d, and phages that specifically bound to M2-i3d were retrieved and isolated. Ninety-six randomly chosen colonies were grown for the expression of their scFvs as soluble proteins. Crude periplasmic extracts were assessed using liposome ELISA, and 32 extracts recognized the M2-i3d-proteoliposomes, but not the empty liposomes (Fig. 1B). Sequence analysis revealed only one scFv (Fig. 2). This scFv, designated as 18A107, was recloned for large scale expression, purification, and characterization.

Binding kinetics

In SPR assays using Biacore, knowledge of the analyte concentration is a prerequisite for a detailed kinetic analysis. The purified GPCR was available in the present study, and using a crude *E.coli* periplasmic extract was sufficient for immobilizing the scFv on the sensor chip (e.g. an immobilized anti-HA antibody could also be used to capture the scFv with a HA tag at the C-terminus). Therefore, we devised a SPR assay in which purified M2-i3d was flowed over the immobilized scFvs in detergent-containing buffer (Fig. 3A).

Approximately 2000 RU of the biotinylated anti-HA antibody was immobilized on the surfaces of both flow cells 1 and 2. The scFv 18A107-containing crude periplasmic extract was injected over the predefined anti-HA immobilized sensor surface (flow cell 2), which resulted in capture responses of approximately 150 RU. QNB-bound M2-i3d at concentrations of 0, 1, 2, 4, or 8 $\mu\text{g/mL}$ was flowed over both flow cells. The kinetic

parameters for scFv18A107 with QNB-bound M2-i3d interactions were examined (Fig. 3B). ScFv 18A107 showed high affinity to QNB-bound M2-i3d, $K_D = 1.0$ nM, with $k_{on} = 1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{off} = 1.5 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$.

Epitope mapping

GPCRs have seven hydrophobic transmembrane helices and eight hydrophilic extramembrane regions, and anti-GPCR antibodies should bind to one or more of the extramembranous parts of GPCRs. To identify the epitope of scFv 18A107, each polypeptide segment located within the extracellular N-distal or intracellular C-distal domains or within the extra- or intracellular loops of M2-i3d was replaced with an arbitrary sequence to construct M2-Nr, M2-i1r, M2-o1r, M2-i2r, M2-o2r, M2-i3r, M2-o3r, and M2-Cr variants (Table 1). The expression of the resultant variants in *S. cerevisiae* was verified using the in-gel fluorescence of GFP as described⁽¹⁷⁾ (data not shown). Aliquots of membrane fractions from these variants were directly immobilized on a MaxiSorp plate to perform an ELISA. ScFv 18A107 could recognize these variants, except for M2-i3r (Fig. 4A), suggesting that the epitope is located within the ICL3.

Detailed mapping of the epitope was conducted by alanine scanning mutagenesis within the ICL3 (Table 1). The amino acid substitutions, K219A, D220A, K222A, E223A, P224A, V225A and N227A resulted in the significant loss in the reactivity of scFv 18A107 (Fig. 4B). These results suggest that the side chain of the residue Lys221 may not have contributed to the contact with scFv 18A107. Similar results were obtained in western-blotting experiments using SDS-denatured variant proteins (data

not shown). Thus, the epitope of scFv 18A107 could be defined as a peptide segment from Lys219 to Asn227 within the ICL3. This amino acid sequence does not exist in the other subtypes of muscarinic receptor (Fig. 4C), suggesting the high specificity of scFv 18A107 on the M2 receptor.

Discussion

Human and mammalian GPCRs typically exhibit limited thermal stability once solubilized from the hydrophobic environment of the lipid bilayer by a detergent. Thus, the use of detergent-solubilized purified GPCRs as binding targets may lead to difficulties in maintaining the structural integrity of the GPCRs during the antibody selection process, and additional intricate strategies may be required to exclude false positives that recognize denatured GPCRs and their aggregates. In contrast, proteoliposome-targeted strategies have been used in anti-GPCR antibody screening via traditional hybridoma technology to successfully identify monoclonal antibodies against the human β_2 adrenergic receptor (β_2 AR),^(21,22) human orphan receptor RAI3 (also known as GPRC5A and RAIG-1),⁽²³⁾ and human adenosine A_{2A} receptor (A_{2A} R).⁽²⁴⁾ Fab fragments to the β_2 AR and A_{2A} R were used as “crystallization chaperones”^(25,26,27,28) to facilitate structural determination.

In this context, we utilized this proteoliposome-based strategy to devise an efficient method for anti-GPCR antibody production via phage display technology. Using the human M2 receptor as a model target, we succeeded in isolating of a high-affinity Fv fragment without further affinity maturation procedures. The whole process, from the immunization to the isolation of a single scFv, was completed within a short period of time (~10 weeks). To the best of our knowledge, this is the first study to demonstrate proteoliposome-targeted selection from a focused phage library built from immunized mice to isolate anti-GPCR antibodies. A similar approach has been used for the non-GPCR target, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

(AMPA)-selective glutamate receptor GluRD,⁽²⁹⁾ which has large extramembraneous hydrophilic domains unlike GPCRs. The procedures we established in this study may be useful for obtaining specific binders against human and mammalian GPCRs as well as other integral membrane proteins, especially those with low thermal stability.

The selected recombinant antibody fragment, Fv 18A107, could recognize the M2 receptor in its detergent-solubilized form as well as that embedded in lipid bilayers. Due to this versatility and high affinity for the M2 receptor, this Fv can be used in various applications such as purification, immunoprecipitation or immunohistochemistry. Crystallization trials of M2-i3d complexed with Fv 18A107 were unsuccessful despite extensive screening, and this may have been because binding via a linear epitope does not stabilize one specific conformation of the M2 receptor. The epitope was located within the ICL3, which is known to be important for G-protein activation, and Fv 18A107 may have prevented coupling of the M2 receptor to the G protein due to steric competition. The intracellular expression of scFv18A107 as an “intrabody”⁽³⁰⁾ may become a novel type of antagonist that inhibits signaling pathways downstream of G-protein activation.

In conclusion, we obtained a high-affinity recombinant scFv against the human M2 receptor using proteoliposome-based technology with no further *in vitro* affinity maturation, which demonstrated that our approach can be used to generate binders to GPCRs and other integral membrane proteins.

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Figure Legends

Fig. 1. Proteoliposome-targeted ELISA (liposome ELISA) to test the binding of selected scFv clones after recursive phage display cycles. (A) Schematic representation of the experimental set-up. Biotinylated liposomes were captured in streptavidin-immobilized ELISA plates, and *E.coli* periplasmic extracts containing HA-tagged scFv for each clone were added. ScFv binding was detected with an anti-HA IgG-horseradish peroxidase conjugate. (B) Representative results of liposome ELISA. Solid bar: M2-i3d proteoliposome-targeted signal (binding); open bar: empty liposome-targeted signal (background). Clones marked with an asterisk were selected as positives and sequenced.

Fig. 2. Nucleotide and amino acid deduced sequences of the scFv 18A107. The two SfiI restriction sites are represented in gray. The complementarity determining region (CDR) of the 18A107 antibody according to the Kabat numbering are underlined, and the 18-residue linker between the light and heavy variable domains is represented in italic.

Fig. 3. SPR analysis of the interaction between scFv 18A107 and detergent-solubilized M2-i3d. (A) Capture assay format used on the Biacore T100. Biotinylated anti-HA IgG (B-IgG) was immobilized in both flow cells 1 and 2 of a streptavidin (SA)-coated sensor chip. ScFvs (scFv-HA) were subsequently captured in flow cell 2. Flow cell 1 was used as a reference surface. M2-i3d was passed over each flow cell simultaneously

for 2 min, and the dissociation of M2-i3d in buffer was monitored for 5 min. (B) Sensorgrams (red) and fitted curves (black) for M2-i3d binding to captured scFv clone 18A107. Detergent-solubilized QNB-bound M2-i3d was injected at 1, 2, 4, or 8 $\mu\text{g/mL}$, and the data were fit to a 1:1 model.

Fig. 4. Mapping of the scFv 18A107 epitope on M2-i3d by crude membrane-targeted ELISA. (A) Identification of the critical hydrophilic domains of M2-i3d for binding to the scFv. To facilitate an interpretation, ELISA signals were background-subtracted and normalized to M2-i3d reactivity with the Fv. Results are the mean \pm s.d. from three independent experiments. Mock: reactivity against the membrane fraction from yeast cells harboring the pDDGFP-2 empty vector. (B) Detailed mapping of the epitope. Immunoreactivities of the scFv against a series of M2-i3d variants containing alanine-scanning mutations within the ICL3 are shown. Error bars, s.d.; $n=3$. (C) Sequence alignments of a segment within the ICL3 of the five subtypes of human muscarinic acetylcholine receptors and mammalian counterparts of the subtype M2. The numbers correspond to the residue number used in the UniProt data P08172. The epitope recognized by Fv 18A107 is underlined.

Table 1. M2 receptor variants used for epitope mapping

Variant	Description	Position of mutated segment or residue ^a	Original sequence or residue in M2-i3d	Replaced sequence or residue
M2-Nr	Replacement in the extracellular N-distal domain	2-22	DDSTDSSDNSLALTSPYKTFE	TDDPLGGHTVWQ
M2-i1r	Replacement in the ICL1	51-59	NRHLQTVNN	DKQLKTVDD
M2-o1r	Replacement in the ECL1	86-95	IGYWPLGPVV	MNRWALGNLA
M2-i2r	Replacement in the ICL2	124-139	CVTKPLTYPVKRTTKM	SITRPLTYRAKRSTKR
M2-o2r	Replacement in the ECL2	164-183	FIVGVRTVEDGECYIQFFSN	YFVGKRTVPPGECFIQFLSE
M2-i3r	Replacement in the ICL3	215-383 (234-381 deleted)	SRIKKDKKEPVANQDPVSPEK	RQLQKIDKSEGRFHVQNLSQVEQDG RTGHGLRRSSKFCLKEH
M2-o3r	Replacement in the ECL3	414-419	APCIPN	DSCVPK
M2-Cr	Replacement in the intracellular C-distal domain	445-466	ATFKKTFKHLMLCHYKNIGATR	KT
S215A	Alanine scanning mutagenesis within ICL3	215	S	A
R216A		216	R	A
I217A		217	I	A
K218A		218	K	A
K219A		219	K	A
D220A		220	D	A
K221A		221	K	A
K222A		222	K	A
E223A		223	E	A
P224A		224	P	A
V225A		225	V	A
N227A		227	N	A
D229A		229	D	A
V231A		231	V	A

^aAmino acid residues were numbered according to the same numbering scheme for full-length sequence of human muscarinic acetylcholine receptor M2 (UniProt # P08172).

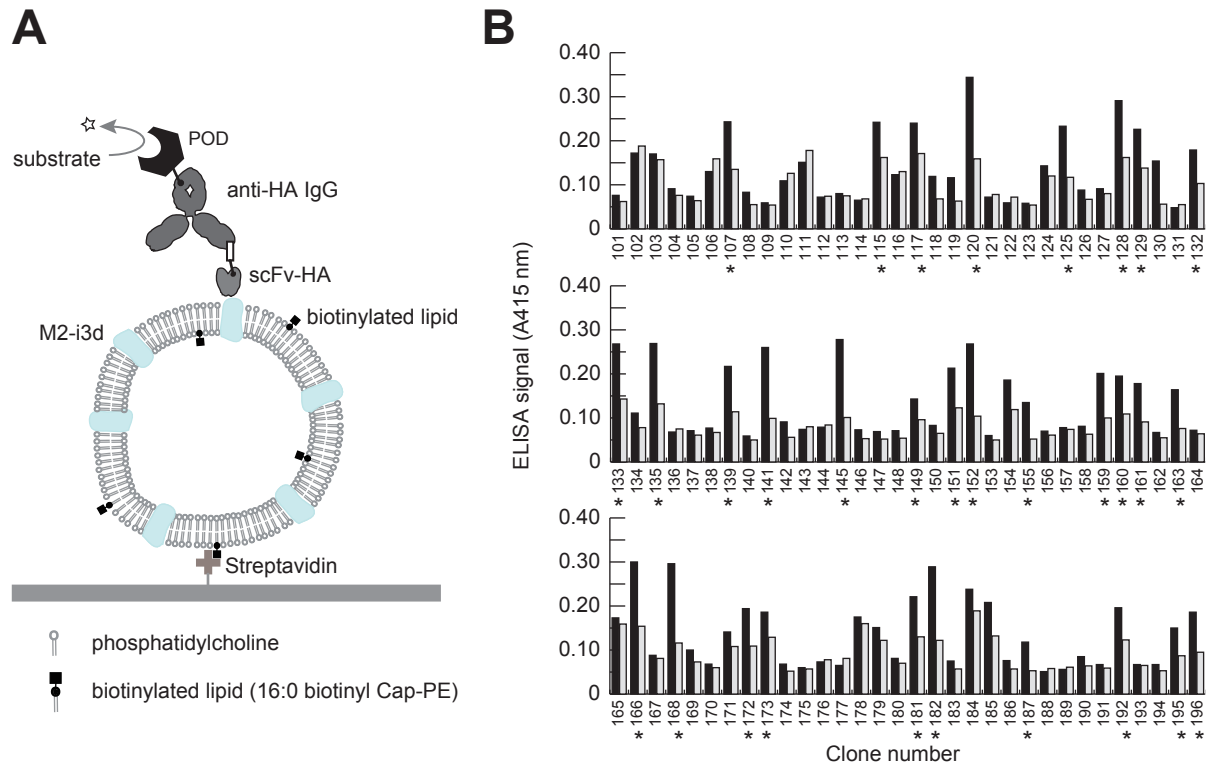


Fig. 1 Suharni et al.

Sfil

GTG GCC CAG GCG GCC GAG CTC GAT ATT GTG ATG ACA CAG TCT CAA AAA TTC ATG TCC ACA < 60
V A Q A A E L D I V M T Q S Q K F M S T

TCA GTA GGA GAC AGG GTC AGC ATC ACC TGC AAG GCC AGT CAG AAT GTG GGT ACT GCT GTA < 120
S V G D R V S I T C K A S Q N V G T A V

CDR-L1

GCC TGG TAT CAA CAG AAA CCA GGA CAA TCT CCT AAA CTA CTG ATT TAC TCG GCA TCC AAT < 180
A W Y Q Q K P G Q S P K L L I Y S A S N

CDR-L2

CGG TAC ACT GGA GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC < 240
R Y T G V P D R F T G S G S G T D F T L

CDR-L2

ACC ATC AGC AAT ATG CAG TCT GAA GAC CTG GCA GAT TAT TTC TGC CAG CAA TAT AGC AGC < 300
T I S N M Q S E D L A D Y F C Q Q Y S S

CDR-L3

TAT CCT CTC ACG TTC GGT GCT GGG ACA AAG TTG GAA ATA AAA TCC TCT GGT GGC GGT GGC < 360
Y P L T F G A G T K L E I K S S G G G G

CDR-L3

TCG GGC GGT GGT GGG GGT GGT TCC TCT AGA TCT TCC CTC GAG GTT CAG CTC CAG CAG TCT < 420
S G G G G G G S S R S S L E V Q L Q Q S

GGG GCA GAA CTT GTG AAG CCA GGG GCC TCA GTC AAG TTG TCC TGC ACA GCT TCT GGC TTC < 480
G A E L V K P G A S V K L S C T A S G F

AAC ATT AAA GAC CAC TTT TTG CAC TGG GTG AAG CAG AGG ACT GAA CAG GGC CTG GAG TGG < 540
N I K D H F L H W V K Q R T E Q G L E W

CDR-H1

ATT GGA AGG ATT GAT CCT GAG GAT GGT GAA ACT AAA TAT GCC CCG AAA TTC CAG GGC AAG < 600
I G R I D P E D G E T K Y A P K F Q G K

CDR-H2

GCC ACT ATT ACA GCA GAC ACA TCC TCC AAT ACA GCC TCC CTG CAG CTC AGC AGC CTG ACA < 660
A T I T A D T S S N T A S L Q L S S L T

TCT GAG GAC ACT GCC GTC TAT TAT TGT GCT ATT ATA CGA GGG AGA AGT TAT TCT ATG GAC < 720
S E D T A V Y Y C A I I R G R S Y S M D

CDR-H3

TAC TGG GGT CAA GGA ACT TCA GTC ACC GTC TCC TCA GCC AAA ACG ACA CCC CCA TCT GTC < 780
Y W G Q G T S V T V S S A K T T P P S V

Sfil

ACT AGT GGC CAG GCC GGC CAG < 801
T S G Q A G Q

Fig. 2 Suharni et al.

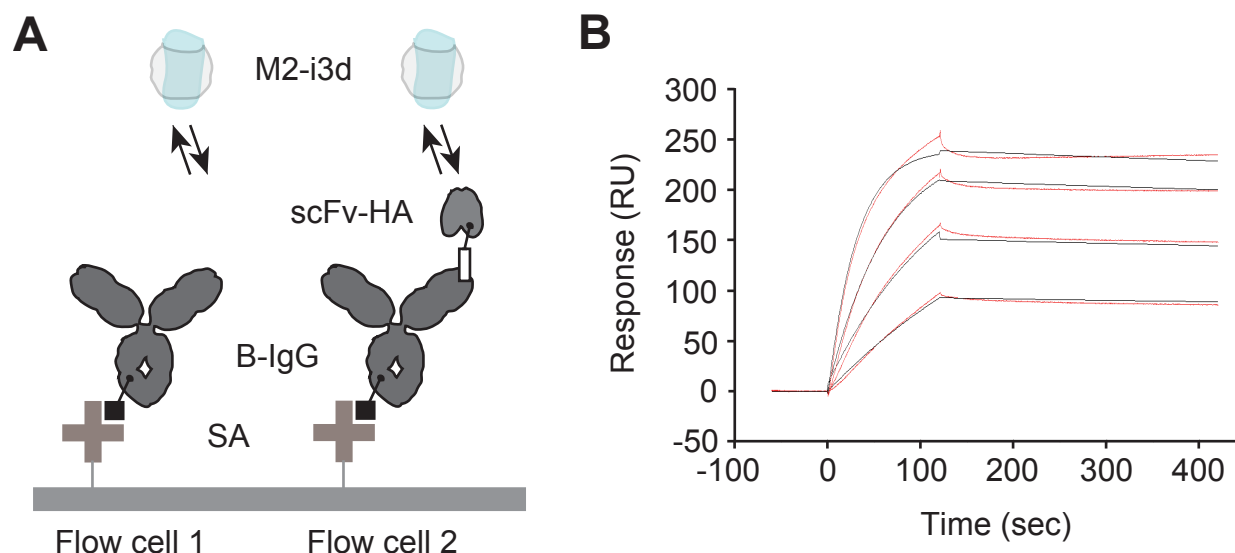


Fig. 3 Suharni et al.

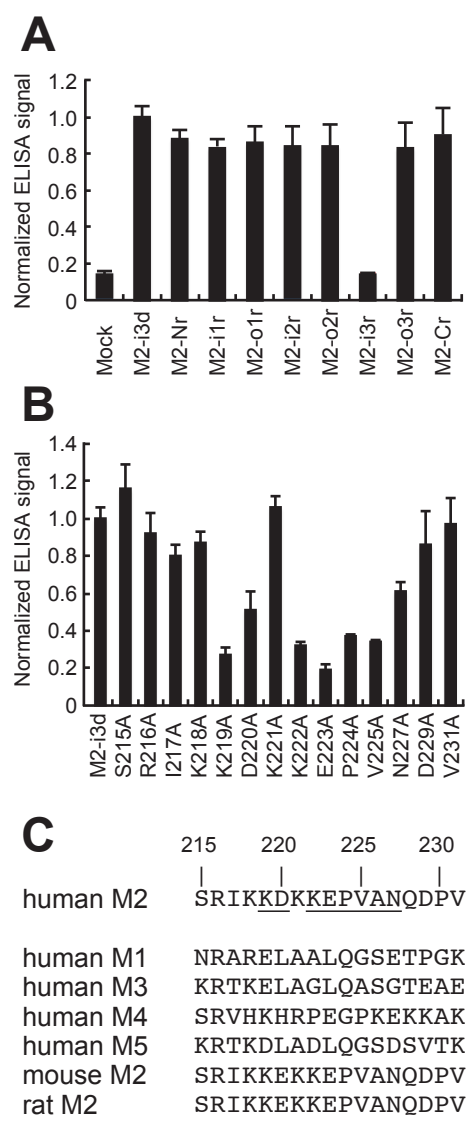


Fig. 4 Suharni et al.